A leap into the unknown

Trends in Parasitology

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REVIEWS

Review

A Leap Into the Unknown – Early Events in African Trypanosome Transmission

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African trypanosomes are mainly transmitted by tsetse flies. In recent years there has been good progress in understanding how the parasites prepare for transmission, detect their changed environment through the perception of different environmental cues, and respond by changing their developmental gene expression. In this review, we discuss the different signals and signaling mechanisms used by the parasites to carry out the early events necessary for their establishment in the fly. We also compare *Trypanosoma brucei* and *Trypanosoma congolense*, parasites that share a common pathway in the early stages of fly colonization but apparently use different mechanisms to achieve this.

Highlights

African trypanosomes are mainly transmitted by tsetse flies.

During uptake in a blood meal, *T. brucei* can use several potential environmental cues to stimulate the initiation of differentiation.

Signal transduction involves a phosphatase signaling cascade and spatial control of key regulatory proteins.

Once differentiation is initiated, several gene regulatory processes control the differentiation from bloodstream to tsetse midgut procyclic forms.

T. brucei and *T. congolense* show a similar route of establishment in the tsetse gut but show different developmental mechanisms.

Early African Trypanosome Transmission Events

African trypanosomes are single-celled parasites that are unusual in having the ability to infect a variety of mammalian hosts, including humans, livestock, and game animals. Here, they cause human African trypanosomiasis (HAT) and animal African trypanosomiasis, respectively, the latter being responsible for significant restrictions in economic productivity across swathes of Africa. The host promiscuity of African trypanosomes is not, however, matched by promiscuity in the arthropod vector they use to achieve their transmission. For most of the major African trypanosome species, tsetse flies are an essential component of their life cycle, although different parasite species show different routes through this vector. Thus, *Trypanosoma vivax* is restricted to the mouthparts of the tsetse fly (although non-tsetse mechanical transmission can also occur outside Africa). In contrast, *T. congolense* and *T. brucei* develop within the gut of the fly before eventual passage to the mouthparts and salivary glands, respectively (Box 1). In this review we discuss the regulatory events that contribute to the ability of trypanosomes to perceive their new environment and adapt as they transition from the mammalian bloodstream to the tsetse fly vector. Most molecular knowledge is based on studies in *T. brucei*, but recent discoveries, focused on the adaptations of *T. congolense*, are also described and compared with the events characterized in *T. brucei*. Events relating to the physiological passage and coordinated motility of the parasites within the tsetse fly are not covered here. The picture emerging of trypanosome transmission events is of a sophisticated environmental sensing response that culminates in gene expression changes, with key molecular players at each step having been identified and characterized. In this review we detail these signals, regulators, and regulatory responses.

Preadaptation of Bloodstream-form Parasites for Tsetse Uptake

In the mammalian bloodstream *T. brucei* exists as either a slender or stumpy morphotype, the transition between these forms being signaled by a quorum sensing (QS) mechanism reviewed elsewhere [1]. These morphotypes are not present in *T. congolense* but nonetheless these parasites also demonstrate density-dependent growth arrest. In both parasite species, the arrest supports infection chronicity and, in *T. brucei,* it is also believed to promote transmission because stumpy forms are preadapted to the drastically different environment in the tsetse fly. Indeed, quantitatively, stumpy forms are more robust than slender forms and are able to withstand the changes in pH and proteolytic exposure that they are likely to be encountered upon uptake in a

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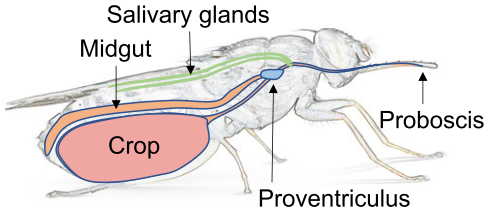
Box 1. Different Paths through the Tsetse Fly in Different Trypanosome Species

T. congolense, like *T. brucei*, first colonizes the tsetse midgut. Subsequently, however, epimastigote and metacyclic forms are found in the proboscis whereas equivalent *T. brucei* stages are found in the salivary glands (Figure I). *T. vivax*, on the other hand, completes development within the fly mouthparts and can also be transmitted mechanically by other biting insects, expanding its range outside Africa [73].

Reports of morphological heterogeneity during infections exist [74,75] but *T. congolense* lacks a morphologically stumpy form, and there is conflicting evidence whether tsetse infectivity correlates with infection stage [75]. Nonetheless, mitochondrial activity is greater in *T. congolense* and *T. vivax* bloodstream forms than in *T. brucei* slender forms [76], and evidence suggests that *T. congolense* has a different strategy to *T. brucei* to prepare for transmission. Thus, *T. congolense* parasites accumulate in G1/G0 of the cell cycle [77], similar to *T. brucei* and *T. vivax* [78], but gene expression changes accompanying the transition from ascending to peak parasitemia in *T. congolense* do not mirror the slender-to-stumpy transition in *T. brucei* [79]. Indeed, *T. congolense* transcripts upregulated at peak parasitemia frequently belong to *T. congolense*-specific groups, such as surface phylome family 22 members [79]. Orthologues of PAD proteins are identifiable in *T. congolense,* with up to 73% amino acid sequence identity, although their role in development is not defined; PAD orthologues are not identifiable in *T. vivax* [46] where tsetse development is restricted to the mouthparts [80].

Transcripts with increased abundance in *T. congolense* insect stages relative to bloodstream stages include those involved in oxidative phosphorylation and amino acid metabolism [81], matching *T. brucei* and *T. vivax* [82]. However, proteomic data sets of developmental regulation show limited correlation between *T. congolense* and *T. brucei* [83,84]. Three sets of *T. congolense* surface molecules characterize tsetse stages: GARP (glutamic acid/alanine-rich protein) [85,86], a protease-resistant surface molecule (PRS) [87], and *T. congolense* procyclins [88]. *T. congolense* procyclins are expressed by midgut forms. They are heavily glycosylated and comprise heptapeptide repeats (EPGENGT) reminiscent of EP or GPEET repeats within *T. brucei* procyclins. Temporally, PRS and procyclins appear first, and then GARP. *T. vivax* lacks procyclins, but several *T. vivax* developmentally regulated surface families have been identified, including a family related to *T. congolense* GARP [82].

(A)



(B)

	T. brucei	*T. congolense*
Morphotypes in host	Pleomorphic (slender, intermediate, stumpy)	Monomorphic
Quorum sensing	Density-dependent stumpy formation	Density-dependent G1 arrest
Quorum sensing	QS pathway genes present	QS pathway genes present
Surface molecule remodeling	PAD enrichment in stumpy forms	Family 22 and other surface phylome members enriched at peak parasitaemia
Occupied digestive tract location in the vector	Crop; Midgut and proventriculus; Salivary gland	Crop; Midgut and proventriculus; Proboscis

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Figure I. Differences between *Trypanosoma brucei* and *Trypanosoma congolense* in Their Transmission through the Tsetse Fly. (A) Relevant components of the tsetse digestive tract are shown, highlighting the position of the fly proboscis, crop, midgut, and salivary glands. (B) Comparison between the key features of *T. brucei* and *T. congolense* transmission biology highlighting their relative morphological heterogeneity in the bloodstream, their ability to arrest in response to parasite density, and the integrity of the components required for this signaling response. Identified molecules enriched in arrested bloodstream forms (stumpy forms for *T. brucei*, peak parasitemia forms for *T. congolense*) are also shown. Finally, the journey of each parasite species within the fly is shown. Abbreviations: PAD, protein associated with differentiation; QS, quorum sensing.

Glossary

Epigenetic control: changes in gene regulation controlled by heritable DNA modifications (e.g., acetylation, methylation) that affect chromatin organization.
Monomorphic: refers to parasites that have a reduced ability to generate stumpy forms after serial syringe passage between hosts (i.e., without tsetse), or after long-term *in vitro* culture.
Pleomorphic: refers to those parasites that are able to generate bloodstream slender, intermediate, and stumpy forms. Slender forms are proliferative, whereas stumpy forms are nonproliferative and express the PAD1 marker. Intermediate cells are not clearly defined morphologically but have committed to become stumpy forms. This might involve further cell division after commitment.
Post-transcriptional regulation: changes in mRNA abundance generated by regulated stability or instability of the mRNA, often through interaction with RNA-binding proteins. Control can also operate at the level of protein synthesis or turnover (this representing post-translational regulation).
Protein kinase: an enzyme that phosphorylates itself or another protein, this often changes its activity or interactions.
Protein phosphatase: an enzyme that removes a phosphate group from proteins, this often changes its activity or interactions.
RNAi: RNA interference, a gene-silencing mechanism that can be used to experimentally deplete a transcript in the trypanosome.
Stumpy regulatory nexus (STuRN): a site close to the flagellar pocket, in stumpy forms, where regulatory molecules gather.

tsetse blood meal [2,3]. They are also less susceptible to antibody-mediated destruction [4]. With respect to metabolic preparation for development, stumpy forms show a partially elaborated mitochondrion [5] and can utilize α-ketoglutarate as an alternative to glucose as an energy source [6–8]. They are also more tolerant of glucose depletion than slender forms, which are quickly killed [9]. 'Protein associated with differentiation' (PAD) proteins (additional abbreviations and gene identifiers are detailed in Table 1) are also upregulated in stumpy forms [10], as are the transcripts for major surface metalloprotease-B (MSP-B), associated with the variable surface glycoprotein (VSG) cleavage necessary for the change of surface coat upon differentiation to procyclic forms [11] as well as transcripts for proteins involved in cytoskeletal remodeling and membrane protein and lipid remodeling [12,13]. Thus, stumpy cells are well adapted to endure uptake and differentiation within the tsetse.

The Initiation of Differentiation

Signaling via Citrate/Cis-Aconitate

The best characterized requirements for the initiation of differentiation are the combination of temperature reduction and sensitivity to the tricarboxylates citrate and/or cis-aconitate (CCA) [14] (Figure 1). These cues are conveyed via the carboxylate transporters comprising the PAD family [10]. The PAD1 protein is already expressed in stumpy forms but, at the lower temperatures encountered upon transmission to the tsetse midgut, a second family member, PAD2, is upregulated and trafficked to the cell surface. These PAD proteins sensitize the parasite to physiological levels of citrate in the blood meal; indeed, their depletion by **RNAi** (see Glossary) renders the parasites less responsive to the CCA differentiation signal [10].

The premature differentiation of stumpy forms in the bloodstream is prevented by a negative regulator, protein tyrosine phosphatase 1 (TbPTP1) [15]. TbPTP1 dephosphorylates its substrate, TbPIP39 [16], this substrate also acts to reinforce the activity of TbPTP1, thereby further inhibiting differentiation. TbPIP39 belongs to the DxDxT class of **protein phosphatases** and displays unusual and important molecular characteristics, including a predicted citrate-binding pocket and a carboxy terminal peroxisomal targeting signal 1 (PTS1). The PTS1 signal marks proteins for sequestration in glycosomes, trypanosome-specific peroxisome-like organelles which act as the site of glycolysis and other metabolic processes [17]. Interestingly, the interaction between TbPTP1 and its substrate, TbPIP39, seems to occur at a specialized site in stumpy forms, the so-called **'stumpy regulatory nexus' (STuRN)**, which is positioned close to a flagellar pocket endoplasmic reticulum (ER) contact site [18] (Figure 1).

Structural predictions and biochemical evidence suggest that the activation of TbPTP1 by TbPIP39 is inhibited by citrate [19]. Citrate, therefore, increases levels of phosphorylated and active TbPIP39, which dissociates from the TbPTP1–TbPIP39 complex. Within 30 min of the initiation of differentiation, TbPIP39 relocates from the STuRN to newly formed glycosomes. Here, it is no longer accessible to inhibition by dephosphorylation mediated by cytosolic TbPTP1, such that the separation of the key regulatory molecules enforces a 'point of no return' in the regulation of trypanosome differentiation. Hence, the coassociation and disassociation of TbPTP1 and TbPIP39 at the STuRN provides spatial control of the responsiveness to the differentiation signal, which ensures the irreversibility of differentiation after initiation. The relocation of TbPIP39 to glycosomes may also initiate the metabolic reprogramming that accompanies differentiation (see later).

Alternative Signaling Pathways

In addition to CCA, other triggers of trypanosome differentiation have been reported: (i) mild acid treatment [20], (ii) brief exposure to proteases [21–23], and (iii) reduced available glucose levels,

Table 1. Molecules and Chemicals Mentioned in the Text

Abbreviation	Description	Gene identifier	Function
ALBA1 ALB2 ALBA3 ALBA4	'Acetylation lowers binding affinity' domain containing proteins	Tb927.11.4460 Tb927.11.4450 Tb927.4.2040 Tb927.4.2030	Developmental RNA regulation
BDF2 BDF3	Bromodomain factor 2 and 3	Tb927.10.7420 Tb927.11.10070	Epigenetic factors
CCA	Citrate/cis-aconitate		Chemicals that stimulate differentiation of *Trypanosoma brucei* from bloodstream to procyclic forms *in vitro*
DOT1B	Disruptor of telomeric silencing factor 1B, a H3 lysine-79 specific histone-lysine N-methyltransferase	Tb927.1.570	Epigenetic factor
EP procyclin	Surface coat protein of *T. brucei* procyclic forms with an internal EP amino acid repeat	Tb927.6.520, Tb927.6.450, Tb927.10.10250, Tb927.10.10260	*T. brucei* surface protein
GARP	Surface coat protein of *Trypanosoma congolense* insect forms	TcIL3000_0_60810 TcIL3000_0_60820 TcIL3000_0_60830 TcIL3000_0_60840 TcIL3000_0_60850	*T. congolense* surface protein
GPEET procyclin	Surface coat protein of *T. brucei* procyclic forms with an internal GPEET amino acid repeat	Tb927.6.510	*T. brucei* surface protein
MAPK5	MAP kinase member 5	Tb927.6.4220	Protein kinase
MKK1	MAP kinase kinase kinase 1	Tb927.3.4860	Protein kinase
MSP-B	Major surface metalloprotease-B	Tb927.8.1610 Tb927.8.1620 Tb927.8.1630 Tb927.8.1640	Protease involved in surface coat release
NRK	NIMA-related kinase	NRKA Tb927.4.5390 NRKB Tb927.8.6930	Kinase regulating differentiation to procyclic forms in *T. brucei*
PAD1 PAD2	Protein associated with differentiation 1 and 2	Tb927.7.5930 Tb927.7.5940	Surface transporter involved in recognition of tsetse uptake
PIP39	Protein tyrosine phosphatase 1 interacting protein 39 kD	Tb927.9.6090 Tb927.9.60100	Protein phosphatase that promotes bloodstream-procyclic form differentiation
PTP1	Protein tyrosine phosphatase 1	Tb927.10.6690	Protein phosphatase that inhibits bloodstream-procyclic form differentiation
RBP6 RBP10	RNA-binding protein 6 and 10	Tb927.3.2930 Tb927.8.2780	RNA regulation
RDK1 RDK2	Regulator of differentiation kinase 1 and 2	Tb927.11.14070 Tb927.4.5310	Protein kinase involved in bloodstream-procyclic form differentiation
REG9.1	Regulator of ESAG9 factor 1	Tb927.11.14220	Negative regulator of stumpy-enriched transcripts
VSG	Variable surface glycoprotein		Major surface protein on bloodstream trypanosomes
ZC3H18	CCCH containing zinc-finger protein 18	Tb927.7.2140	Developmental RNA regulation
ZFP1 ZFP2 ZFP3	CCCH containing zinc-finger proteins 1, 2, and 3	Tb927.6.3490 Tb11.01.6590 Tb927.3.720	Developmental RNA regulation

either by using glucose-depleted media [9,24] or by partially inhibiting glucose uptake in parasites using phloretin or 2-deoxy-D-glucose (2-DOG) [25]. The variation in cell lines and lifeforms used (slender, stumpy, **monomorphic** slender) makes it difficult to judge which of these stimuli may be physiologically relevant, particularly for cultured parasites where it can be difficult to

disentangle the kinetics of differentiation events, the continued growth of bloodstream cells, and the outgrowth of differentiated procyclic cells. Nonetheless, using the synchronous differentiation of stumpy forms and markers that are now available to monitor differentiation quantitatively, CCA, mild acid conditions and protease treatment have all been shown to be robust stimuli for the transformation to procyclic forms [19,23]. Furthermore, using RNAi-mediated TbPIP39 depletion, it has been possible to evaluate the extent to which different stimuli signal through the TbPIP39-dependent pathway or through an alternative route. Such experiments have shown that differentiation stimulated by CCA or mild acid is reduced by TbPIP39 depletion whereas differentiation stimulated using proteases remains unaffected, suggesting that it operates via a separate branch. Indeed, there is evidence that CCA and protease operate synergistically to promote differentiation [23].

The contribution of the depletion of glucose to the initiation of differentiation remains controversial. Early studies using monomorphic cells demonstrated that replacing glucose with glycerol favored the emergence of procyclic forms. However, this took place over several days [24], and so whether this represented an enhanced initiation of differentiation or selection for the outgrowth of procyclic forms was unclear. Similarly, the use of the glucose uptake inhibitor, 2-DOG, enhanced the expression of some differentiation-related transcripts in cultured bloodstream forms either as a differentiation or stress response [25]. Most recently, in 2018, Qiu *et al.* [9] showed that **pleomorphic** slender- and stumpy-form parasites rapidly deplete glucose

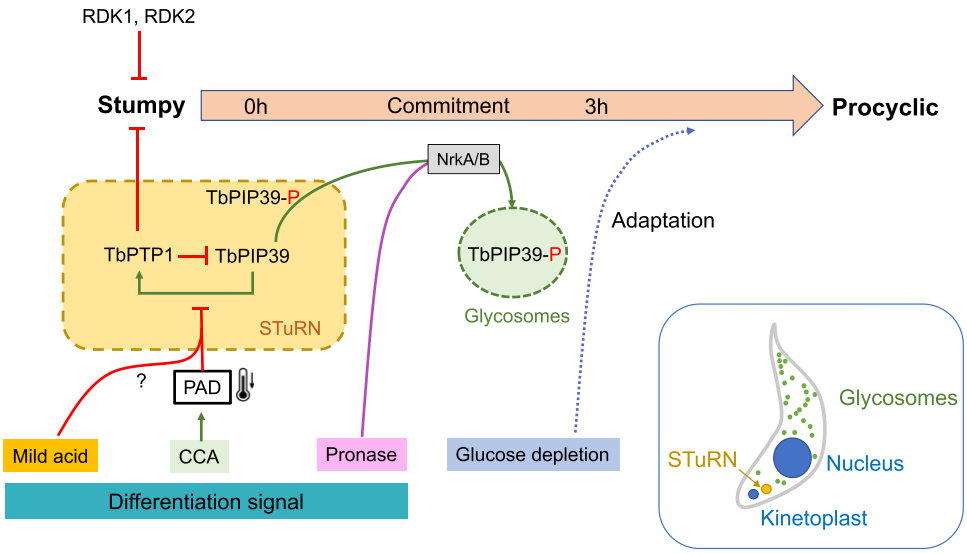

Figure 1. Signaling Events Contributing to the Initiation of Differentiation from Stumpy Forms to Procyclic Forms. Stumpy forms are held poised for differentiation through the action of the protein tyrosine phosphatase, TbPTP1, and the protein kinases RDK1 (regulator of differentiation kinase 1) and RDK2. For the initiation of differentiation to procyclic forms, different stimuli have been reported but the best characterized is mediated through citrate/cis-aconitate (CCA). This is conveyed via 'protein associated with differentiation' (PAD) family proteins. Within the stumpy regulatory nexus (STuRN, positioned close to the stumpy flagellar pocket), copositioning of TbPTP1 and TbPIP39 (TbPTP1 interacting protein, 39 kD) prevents differentiation, with TbPTP1 dephosphorylating and reducing the activity of TbPIP39. In the presence of CCA, this repression is alleviated and TbPIP39 is phosphorylated by an unidentified protein kinase and activated. Within 30 min, TbPTP1 and TbPIP39 redistribute away from the STuRN, with TbPIP39 relocating to glycosomes. Mild acid and pronase can also stimulate rapid differentiation, with mild acid requiring the presence of TbPIP39; protease signaling is TbPIP39-independent. The protein kinase NRKA/B (NIMA-related kinase A and B) is important for differentiation, and in its absence signaling via both CCA and pronase is reduced. Glucose depletion may also stimulate metabolic adaptation of the parasite and eventual outgrowth of adapted procyclic forms. Irreversible commitment to differentiation occurs around 2 h after signal exposure.

from the culture media (from the initial 500 μM to 37–62 μM), leading to the preferential survival of stumpy cells. Furthermore, exposing stumpy cells to 5 μM glucose led to EP procyclin expression and procyclic cell outgrowth over several days, a so-called 'glucose-responsive slow' differentiation response [9]. This contrasts with the rapid differentiation seen after CCA treatment, and there is an absence of any differentiation response over 24 h when parasites are exposed to 50 μM glucose [19] rather than 5 μM (respectively 100× and 1000× lower than physiological bloodstream levels). Interestingly when the transcriptomes of procyclic forms differentiated after glucose starvation (5 μM) or CCA exposure were compared, upregulation of transcripts involved in gluconeogenesis, amino acid metabolism (proline and threonine), and the electron transport chain was detected in cells undergoing 'glucose-responsive slow' differentiation.

In combination, such studies suggest that stumpy cells respond rapidly to CCA, mild acid and proteases, whereas glucose reduction might provoke a slow adaptation and differentiation. Understanding the physiological contribution of glucose depletion to differentiation requires careful kinetic examination of parasite survival and differentiation as well as the analysis of glucose levels in the tsetse fly after a bloodmeal.

Commitment to Differentiation after Initiation of the Process

Once differentiation is stimulated, parasites commit to onward development irrespective of the continued presence of the signal. Exploring this, Domingo-Sananes and her coworkers [26] re-examined earlier studies to investigate the commitment point during the synchronous differentiation from stumpy to procyclic forms [23,27]. This quantitative analysis mapped irreversible commitment to 2–3 h after exposure to CCA, showing that cells after this time not only maintain the expression of procyclin (already expressed at commitment) but also re-enter a proliferative cell cycle (DNA synthesis occurs at 8–10 h after exposure to CCA [28]). When the CCA was removed from the media after the commitment point, these 'CCA-primed cells' completed successful differentiation to procyclic forms, confirming that the cells retained ‘memory’ of their signal exposure. To determine if this signal memory was dependent on new protein synthesis, parasites were incubated with CCA in the presence of the reversible translational inhibitor cycloheximide. Cells with blocked protein synthesis did not respond to CCA; however, removing the inhibitor and re-exposing the parasites to CCA stimulated differentiation with similar kinetics to uninhibited cells. This demonstrated that new protein synthesis (rather than mRNA or post-translational modifications) was needed to retain the memory of signal exposure and also to ‘set the clock’ for the subsequent differentiation events.

Examination of the proteome and phosphoproteome of cells during commitment revealed that changes in protein phosphorylation occurred first (around 1 h after CCA exposure, mainly involving dephosphorylation), followed by large changes in protein expression at 3 h. A key change observed early in differentiation was an upregulation of two serine threonine **protein kinases** encoded by closely related genes, NRKA/NRKB already known to be enriched in stumpy forms [29,30]. Depletion of TbNRKA/B in pleomorphic trypanosomes by RNAi did not affect growth or development in the bloodstream but almost completely inhibited differentiation to procyclic forms. This inhibition operated on both the CCA- and protease-stimulated differentiation pathways, placing the kinase downstream of TbPIP39 in the environmental signaling cascade where the pathways converge (Figure 1). Subsequently, a quantitative label-free proteomic analysis described changes in protein expression through differentiation from short stumpy to procyclic forms [31]. This provided a high-quality temporal map of proteome changes in the early time points of synchronous differentiation from stumpy to procyclic forms adding to earlier studies of the proteome in different developmental forms of the parasite [32–34].

Apart from the described CCA/PAD/TbPTP1/TbPIP39 regulatory cascade, little is known about signaling events regulating life-cycle differentiation. The first kinase identified to regulate developmental competence was a MAP kinase (MKK1) for which null mutants failed to develop salivary gland infections [35]. A further kinase, TbMAPK5, apparently regulates stumpy differentiation *in vivo* [36]. With respect to the differentiation from stumpy to procyclic forms, two regulatory kinases were identified in a kinome-wide RNAi screen: repressor of differentiation kinase 1 and 2 (RDK1 and RDK2) [37]. Although depletion of both kinases promoted spontaneous differentiation to procyclic forms, differences were detected. RDK1 depletion triggered around 20% of cells to differentiate, and this was elevated to 60% when the RNAi cell line was treated with the tyrosine phosphatase inhibitor BZ3 [38], suggesting that RDK1 and TbPTP1 work additively to regulate differentiation. On the other hand, RDK2 seemed to prevent differentiation of all cells within the population [37]. Both kinases were detected in the differentiation proteome. RDK1 was only detectable in established procyclic forms, suggesting an additional, procyclic specific role, in addition to its role regulating differentiation. RDK2, in contrast, was highly expressed in stumpy forms and in the first 12–24 h of differentiation [31], which correlates well with its proposed role. Other kinases and phosphatases that exhibit protein expression profiles suggesting roles in differentiation, include the aforementioned NRKA/B and a putative AGC/RSK family serine/threonine kinase (Tb927.11.5860), both of which are elevated in stumpy forms but then decline during differentiation to procyclic forms. Other putative kinases are either transiently upregulated (Tb927.10.3230) or are only detectable during late time points of the differentiation process (Tb927.4.2680) [31]; both await further exploration.

Gene Regulation before and during Differentiation

Gene regulation is particularly important in trypanosome parasites because of their complex life cycle, the requirement to adapt to different environments or to preadapt in preparation for differentiation. Notably, the parasite's absence of regulatable RNA II polymerase promoters for protein coding genes [39] and their polycistronic transcription requires almost complete reliance on **post-transcriptional** control for regulated gene expression. Supporting this, the genome of trypanosomes contains large numbers of genes encoding predicted RNA-binding proteins [40,41]. To date, many predicted RNA regulators have been studied, and we highlight only a few of those most relevant in the preparation for, and at early stages of, development from bloodstream forms to procyclic forms (Figure 2).

Regulatory Control in the Bloodstream

RBP10

RBP10 is a cytosolic protein with a single RNA recognition motif [42] that seems to be critical for maintenance of parasites as bloodstream forms. Both western blot [43] and proteome analyses [31] indicate that RBP10 is expressed in proliferating bloodstream forms (monomorphic and slender forms) but not in stumpy forms or procyclic forms. When RBP10 is depleted by RNAi in bloodstream forms, the outgrowth of procyclic-form cells is favored, albeit inefficiently in the population. Conversely, with RBP10 ectopic expression in procyclic forms, bloodstream-form-enriched transcripts become more abundant; this results from developmental progression through metacyclic and then bloodstream forms in a subset of the population. The transcripts targeted by RBP10 for translational repression and instability are enriched for the motif $UA(U)_6$, a motif previously identified as enriched in procyclic-form mRNAs [44]. Hence, RBP10 favors maintenance of the bloodstream form state and represses procyclic-enriched gene expression.

REG9.1

The relative control of stumpy- and slender-enriched gene expression represents an interesting regulatory question given that slender and stumpy forms share the same environment (unlike

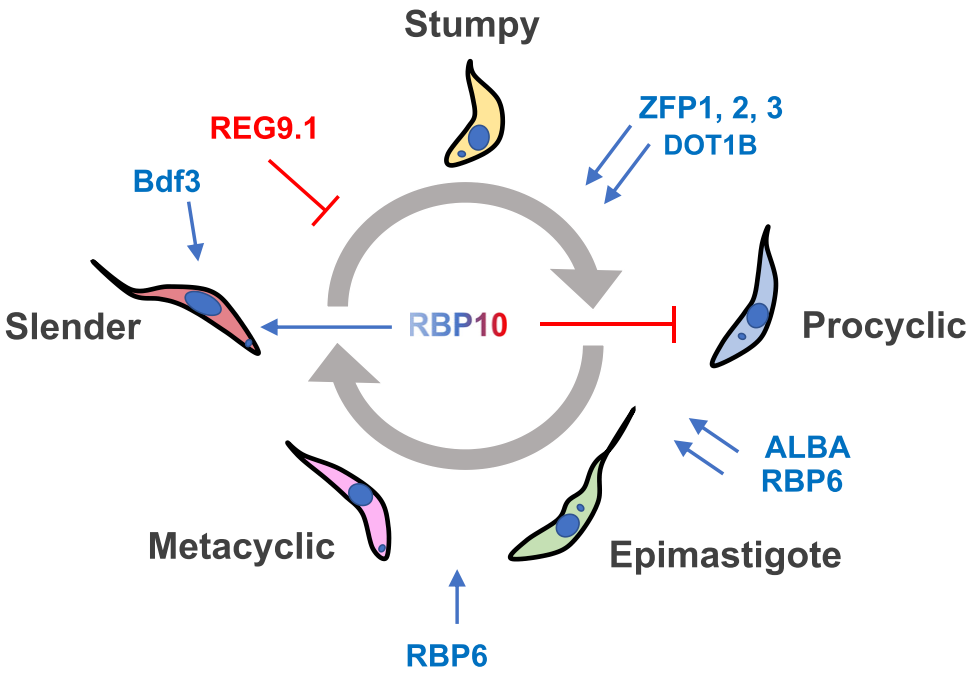

Figure 2. Regulatory Control of the Trypanosome Life Cycle. Different transitions through the parasite's life cycle are depicted with regulators that either promote (in blue) or inhibit (in red) the different developmental transitions being shown. Abbreviations: ALBA, 'acetylation lowers binding affinity' domain-containing protein; BDF, bromodomain factor; DOT1B, 'disruptor of telomeric silencing' factor 1B; RBP, RNA-binding protein; REG9.1, regulator of ESAG 9; ZFP, zinc-finger protein.

bloodstream and procyclic forms). To explore this regulation, a genome-wide RNAi screen was exploited to isolate repressors of stumpy-enriched gene expression [45]. This identified several negative regulators, including one (*REG9.1*; *REG*ulator of ESAG*9*), whose depletion by RNAi up-regulated members of the ESAG9 family. These genes are characterized by their elevated expression in stumpy forms and occasional presence in VSG gene expression sites. Similarly, other stumpy-enriched cell-surface phylome members (Family 5, Family 7) [46] were also upregulated, highlighting the broader role of REG9.1 in the repression of several stumpy-elevated transcripts. Consistent with this role, the depletion of REG9.1 accelerated stumpy formation *in vitro* and *in vivo*, whereas spontaneous differentiation to procyclic forms was observed upon overexpression of the molecule – albeit in only a subset of cells.

Interestingly, while the expression levels of REG9.1 do not change markedly in the life cycle, its subcellular location is regulated. Indeed, the molecule is located at the same paraflagellar site as TbPIP39 and TbPTP1 in stumpy forms, that is, the STuRN [18], but disperses within minutes after the initiation of differentiation. This highlights the importance of the subcellular position of regulatory components in stumpy-form parasites as they prepare for, and initiate, the differentiation events that allow them to adapt to the tsetse midgut environment.

Regulatory Control Early during Differentiation

ZFP Family of Proteins

The first RNA-binding protein identified with a role in regulating stumpy to procyclic differentiation was a small protein, TbZFP1 [47], a member of the CCCH (ZC3H) zinc-finger-motif-containing family [48]. The expression of TbZFP1 was elevated between 2 h and 8 h after the initiation of

differentiation, and then downregulated, reappearing in established procyclic forms [47]. ZFP1 null mutants show compromised kinetoplast repositioning [49], one of the major morphological events marking differentiation between bloodstream and procyclic forms [50].

Another member of the CCCH family, TbZFP2, was also implicated in the control of early differentiation events [47]. The protein is constitutively expressed in both bloodstream and procyclic forms, and during synchronous differentiation between these forms. RNAi targeting TbZFP2 inhibits the initiation of differentiation (as assessed by surface expression of EP procyclin), contrasting with TbZFP1 where surface antigen exchange occurs successfully [47]. When ectopically expressed, TbZFP2 generates a posterior microtubule extension ('nozzle'), this being dependent on the integrity of its CCCH motif [47]. A third member of the CCCH family, TbZFP3 was further identified [51] in a data base search using the TbZFP1 and TbZFP2 protein sequences. TbZFP3 shows an overall motif organization similar to that of TbZFP2, comprising a C-terminal CCCH and an N-terminal WW protein-interaction domain, most similar to a WW domain in E3 ubiquitin ligases. In addition to the aforementioned motifs, TbZFP3 also has three copies of the RGG-predicted RNA-binding motif located between the WW and CCCH domains. The expression profile of TbZFP3 resembles that of TbZFP2, and both proteins associate with the translation apparatus in procyclic forms but not in bloodstream forms [51]. Ectopic overexpression of TbZFP3 in bloodstream forms stimulates differentiation to procyclic forms, complementing the ablation of TbZFP2 by RNAi that inhibits differentiation. Moreover, yeast two-hybrid and coimmunoprecipitation experiments demonstrate that TbZFP1 can interact directly with TbZFP2 and TbZFP3 *in vitro* and *in vivo*, suggesting their overlapping roles and/or involvement in the same protein complex(es).

With respect to mRNA targets, TbZFP3 coassociates with EP1 and GPEET procyclin mRNA, but not with the distinctly regulated transcripts for EP2 and EP3 procyclin [52]. EP1 procyclin regulation is mediated via predicted regions within its 3′UTR, namely the Loop II [53,54] and 16mer sequences that control transcript stability and translation [53]. It was proposed that TbZFP3 competes with a negative regulator targeting the Loop II region, such that its overexpression enhanced EP procyclin expression, this being demonstrated by mass spectrometry. Subsequently, another zinc-finger protein, ZC3H18, with two CCCH motifs, was identified with a potential role in differentiation. Although RNAi depletion of ZC3H18 does not affect viability of the established procyclic cells, it causes a delay in the *in vitro* differentiation of high-density bloodstream forms to procyclic forms.

Regulatory Events at Subsequent Steps in the Developmental Cycle

ALBA Proteins

As well as CCCH-motif-containing molecules, important in the preparation for tsetse uptake and early events thereafter, ALBA ('acetylation lowers binding affinity')-like proteins have been shown to be important in the parasite's development. ALBA proteins comprise a large family known for their eponymous ALBA domain (Pfam PF01918) with nucleic acid–binding ability [55], and which have a general role in the developmental program of several microorganisms [56]. The trypanosome genome encodes four proteins containing ALBA domains, two of which, ALBA3 and ALBA4, show 85% identity at the DNA level. In addition to the ALBA domain, both proteins have multiple RGG repeats at their C terminus, believed to be important in nucleic acid binding. The four ALBA proteins can be found in a complex, with ALBA3 as a core component [57], and the protein family binds mRNAs carrying regulatory elements in their 3′UTR, for example, GPEET procyclin transcripts [57]. Overall, the expression profile and overexpression phenotypes exhibited by ALBA domain proteins suggest a role in

the mesocyclic-to-epimastigote transition, although a role at other transitions cannot be excluded.

RBP6

Remarkably, the onward development from procyclic forms toward infectious metacyclic forms is under dominant control of a single RNA-binding protein, RBP6 [58]. This molecule is normally elevated in proventricular forms of the parasite in the tsetse, but if ectopically overexpressed the molecule precipitates full differentiation to infectious metacyclic forms. Moreover, further direct development to proliferative bloodstream forms is possible through introduction of a single mutation in RBP6 [59].

In combination, trypanosome RNA-binding proteins seem to exert relatively straightforward control over the parasite's life cycle, where the level or (potentially) post-translational modification of a restricted set of individual regulators can drive the activation of coherent developmental transitions involving widespread cellular processes.

The Contribution of Epigenetic Control to Development

As well as changes in gene expression enacted by RNA-binding proteins during development, there is also emerging evidence for a contribution from **epigenetic** events.

DOT1B

DOT1B (Tb927.1.570) is a histone methyltransferase responsible for the methylation of histone H3 on lysine 76 [60]. Although not required in either bloodstream or procyclic forms, it is essential for bloodstream-to-procyclic differentiation [61] and is involved in transcriptional regulation of the expressed VSG in bloodstream forms [62–64]. DOT1B is expressed 12 h after stumpy cells are induced to differentiate with CCA, this coinciding with their entry into the differentiation cell cycle, suggesting a role for DOT1B during or after the first S-phase of differentiation [31]. Consistent with this, DOT1B-deficient stumpy cells treated with CCA undergo the normal early events of differentiation (VSG release, procyclin expression) but then exhibit unusual nuclear/kinetoplast configurations at timepoints after the onset of cell cycle re-entry [31].

Bromodomain Proteins

Bromodomain proteins are epigenetic readers of lysine acetylation that can be targeted by selective inhibitors. Exploiting a particular inhibitor, BET151, Schulz and colleagues [65] observed that bloodstream cells can be induced to express procyclic-form transcripts and undergo differentiation, albeit with low efficiency. These inhibitor studies were supported by the specific RNAi depletion of the transcripts of one of the bromodomain proteins identified in the trypanosome genome, BDF3, suggesting that it may contribute to the maintenance of the bloodstream form state. Depletion of a second bromodomain protein, BDF2, had little effect on procyclic-enriched transcript expression but did affect VSG monoallelic control. The positional redistribution of bromodomain proteins, and the consequent effects on gene regulation, was proposed as a possible mechanism for initiating a procyclic-form-specific transcriptional program during stumpy-to-procyclic-form differentiation.

The Contribution of Metabolic Regulation to Development

Accompanying the regulatory events that drive differentiation from bloodstream to procyclic forms, the parasite also experiences dramatic changes in its metabolic environment with inevitable consequences for the parasite's overall physiology. In the bloodstream, glucose is a dominant carbon source, and slender forms utilize this via glycolysis without mitochondrial involvement.

Glycerol can also be utilized through gluconeogenesis, potentially as an adaptation for tissue compartmentation [66,67]. Stumpy forms, in contrast, can catabolize α-ketoglutarate as an adaptation for the depletion of glucose in a tsetse blood meal. Stumpy forms do not require mitochondrial DNA to be present for their viability; however, their lifespan is reduced in its absence and the defective cells are unable to generate a mitochondrial membrane potential [8]. In procyclic forms, L-proline is actively taken up and catabolized inside the mitochondrion into succinate, alanine, and acetate with the production of intermediate metabolites, reduced cofactors, and ATP. The proline is converted to glutamate in three steps: first, proline is oxidized into Δ1-pyrroline-5-carboxylate (P5C) by a FAD-dependent proline dehydrogenase (TbProDH) [68]. Second, the P5C ring is spontaneously opened to produce glutamate-γ-semialdehyde (γ GS), and finally the γ GS is further oxidized to glutamic acid by a P5C dehydrogenase (TbP5CDH). This enzyme is present in procyclic but not bloodstream forms, and the depletion of TbP5CDH significantly reduces midgut infection rates. These results suggest that, to enable survival in the glucose-scarce environment of the tsetse midgut, procyclic forms rely both on the tsetse fly-derived proline and a fully functional proline catabolic pathway [69]. Proline and glycerol can also feed gluconeogenesis in procyclic forms and this appears to be important for later steps of development of the parasite in the fly [70].

Concluding Remarks

In the past few years there have been substantial strides taken in understanding the molecular events that occur during, and which regulate, the transition from bloodstream to procyclic forms. We now have insight into how the parasite prepares for transmission, perceives its changed environment, and responds through altered gene expression enacted by regulatory RNA-binding proteins, epigenetic changes, and metabolic adaptation. Furthermore, there has been an increasing appreciation that different trypanosome species exhibit different solutions to the problem of tsetse colonization. These differences are highlighted by the presence of morphologically stumpy forms, which represent a *brucei*-specific adaptation not present in either *T. congolense* or *T. vivax*. Whilst it is important to recognize that morphology is not necessarily the most important characteristic of stumpy forms – such that underlying controls in the developmental pathway may well be conserved between species – the molecular comparisons between the species point to important differences that require exploration. Similarly, this review has focused tightly on parasite-specific processes and has not considered interactions with either the host or the tsetse fly except in limited terms. This is clearly naïve given the exquisite sensitivity of trypanosomes to their environment, their sophisticated mechanisms of immune evasion (in the mammal and tsetse), and their movement and coordinated motility within the vector [71,72]. Hence, whilst there is now much that we understand about how the trypanosome can successfully 'leap into the unknown' during its transmission to tsetse flies, there remains a great deal to uncover and important controversies remain (see Outstanding Questions). The tools now available for the genetic manipulation of *T. brucei* and *T. congolense* provide a tractable system for understanding and comparing the differentiation events of the parasites in detail and we can look forward to rapid progress.

Outstanding Questions

How are environmental cues of differentiation transduced via coherent signaling pathways to drive developmental gene expression?

What is the molecular composition and cytological origin of the regulatory STuRN structure?

How does the trypanosome process the multiple signals that stimulate differentiation (e.g., CCA, mild acid, protease exposure) to coordinate its developmental response?

What molecular events define the irreversible commitment to differentiation?

How does metabolic adaptation drive or respond to the differentiation response?

How do gene regulators coordinate their activity to ensure a successful differentiation response at both the level of epigenetic and post-transcriptional control?

Does *T. congolense* have a specialized transmission stage equivalent to the stumpy form of *T. brucei*?

How different are the molecular regulators and events that contribute to the early development of *T. brucei* and *T. congolense* in the tsetse gut?

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